



University of Groningen

Antioxidants, a radical solution?

Janknegt, Paul Johannes

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2009

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Janknegt, P. J. (2009). Antioxidants, a radical solution? Antioxidant responses of marine microalgae to ultraviolet radiation. Groningen: s.n.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy

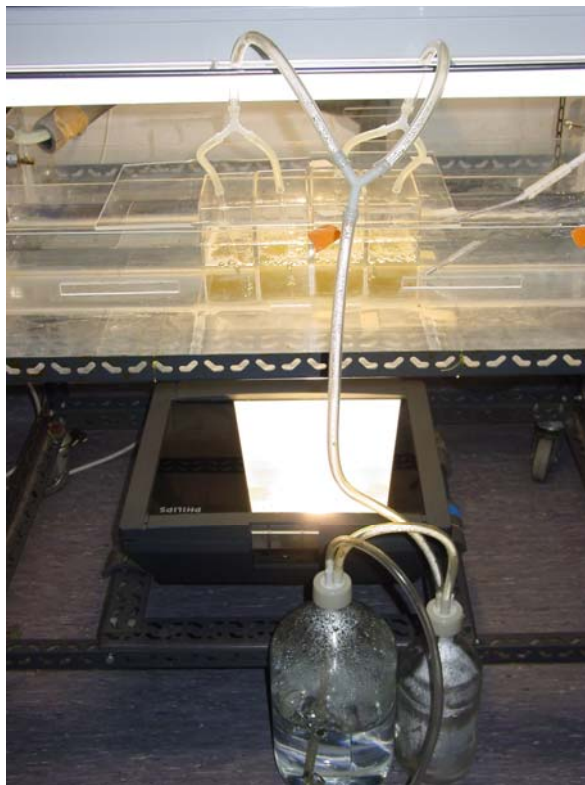
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Chapter 4

Excessive irradiance and antioxidant responses of an Antarctic marine diatom exposed to iron limitation and to dynamic irradiance.

Willem H. Van de Poll, Paul. J. Janknegt, Maria A. Van Leeuwe, Ronald J. W. Visser and Anita G. J. Buma



Published in: *Journal of Photochemistry and Photobiology B: Biology* 94: 32-37 (2009)

Abstract

The synergistic effects of iron limitation and irradiance dynamics on growth, photosynthesis, antioxidant activity and excessive PAR (400-700 nm) and UVR (280-400 nm) sensitivity were investigated for the Antarctic marine diatom *Chaetoceros brevis*. Iron-limited and iron-replete cultures were exposed to identical daily irradiance levels, supplied as dynamic (20-1350 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and constant (260 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) irradiance. After acclimation, growth, maximal quantum yield of PSII (Fv/Fm), pigment composition, and the activities of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) were determined. Then, excessive irradiance sensitivity was assessed by monitoring pigment composition, Fv/Fm and viability loss during a single excessive PAR and UV treatment.

Iron limitation reduced growth rates, Fv/Fm dynamics, and cellular pigments pools. Cellular pigment concentrations were higher under dynamic irradiance than under constant irradiance but this difference was less pronounced under iron limitation compared to iron replete conditions. SOD and APX activities increased during dynamic irradiance under iron limitation, suggesting increased radical formation around PSII. Despite these physiological differences, no effects on growth were observed between constant and dynamic irradiance cultivation in iron-limited and iron-replete cells. The applied culturing conditions did not affect glutathione reductase activity in *C. brevis*. Fv/Fm and xanthophyll de-epoxidation dynamics during excessive irradiance were not different for iron-limited and replete cells and viability loss was not found during excessive irradiance. This study revealed photoacclimation differences between iron-limited and iron-replete *C. brevis* cultures that did not affect growth rates and excessive irradiance sensitivity after acclimation to constant and dynamic irradiance.

Introduction

The combination of iron limitation and deep wind driven vertical mixing can suppress primary productivity in vast parts of the Southern Ocean, leaving much of the dissolved nitrate, phosphate, and silicate unused for algal growth (De Baar *et al.* 2005, Boyd *et al.* 2007). These frequently co-occurring conditions both affect the photoacclimation state of algae. Vertical mixing in the water column can mediate strong irradiance fluctuations, from excessive irradiance near the water surface to complete darkness below the euphotic zone. These dynamics require contrasting acclimation pathways such as increased light harvesting under low irradiance as well as increased photoprotection when irradiance exceeds the photosynthetic requirements (MacIntyre & Geider 1996, Van de Poll *et al.* 2007). The effects of iron limitation on photoacclimation are well documented. When iron becomes limiting, algae respond by reducing the abundance of iron rich cellular components such as cytochrome b_6-f and PSI complexes (Raven 1990, Strzepek & Harrison 2004). This impairs the electron transport capacity during photosynthesis and leads to a strong reduction in cellular light harvesting pigments and decreased growth rates (Geider *et al.* 1993, Van Leeuwe & Stefels 1998).

How iron limitation affects photosynthesis and growth under dynamic irradiance remains unknown. Cytochrome b_6-f complexes are crucial in the buildup of a proton gradient across the thylakoid membrane, which activates protective heat dissipation via the xanthophyll cycle during excessive irradiance. Therefore, it was suggested that iron-limited algae were less able to cope with rapid irradiance fluctuations than those under iron replete conditions (Strzepek & Harrison 2004). Furthermore, Van Leeuwe & Stefels (1998) observed reduced xanthophyll de-epoxidation under high irradiance in iron-limited *Phaeocystis antarctica*. Therefore, a reduction in cytochrome b_6-f complexes may affect the efficiency of the xanthophyll cycle, and thus the protective down regulation of photosynthesis during excessive irradiance. Consequently, iron-limited algae could be more vulnerable to excessive irradiance due to increased formation of reactive oxygen species (ROS). ROS are typically formed as byproducts of electron transport and cellular metabolism. Environmental stress such as excessive PAR and UV exposure near the water surface can significantly enhance ROS formation, inhibiting photosynthesis and threatening viability (Van de Poll *et al.* 2005).

Cellular ROS concentrations are controlled by an elaborate antioxidant network. The superoxide radical is converted enzymatically to H_2O_2 by superoxide dismutase (SOD), which is subsequently neutralized to H_2O by ascorbate peroxidase (APX). Glutathione is used to regenerate ascorbate, which is the substrate of APX in the latter reaction. The redox status of glutathione is regulated by glutathione reductase (GR). Iron limitation was found to increase ROS production and superoxide dismutase (SOD) activity in marine diatoms (Peers & Price 2004). Nevertheless, a preliminary study indicated that iron limited algae were less sensitive to excessive PAR and UV radiation exposure than iron replete algae (Van de Poll *et al.* 2005). Furthermore, numerous studies demonstrated that iron-limited algae accumulated more protective xanthophyll cycle pigments relative to their light harvesting pigments compared to iron-replete cells (Geider *et al.* 1993). This emphasizes that the effect of iron limitation on the regulation of photosynthesis is not completely understood, let alone under dynamic irradiance as experienced in the field. Therefore, the responses of pigments, chlorophyll fluorescence and crucial components of the antioxidant

network were studied for the Antarctic diatom *Chaetoceros brevis* under simulated dynamic and constant irradiance for iron-limited and iron-replete conditions, followed by excessive (UV) irradiance treatments, during which Fv/Fm, xanthophyll cycling and viability were monitored.

Materials and methods

Cultivation and experimental design

Iron replete cultures of *Chaetoceros brevis* (CCMP 163) were grown as batch cultures in autoclaved sea water of 35 PSU enriched with f-2 nutrients (Guillard & Ryter 1962). Iron limited cultures were grown in sea water of 35 PSU, collected from the Southern Ocean, enriched with f-2 nutrients (without iron), and then run over a Chelex-100 column (Chelex, Rochester, NY, USA) to remove iron (except the silicate stock). The polycarbonate cultivation vessels of iron limited cells were washed with 1 N HCl and handled in a clean room to prevent iron contamination, whereas 10 μ M EDTA (final concentration) was added to bind remaining iron. Iron-limited and replete cultures were grown at 4.5°C in a cooled culture cabinet under 75 μ mol \cdot photons \cdot m⁻² \cdot s⁻¹ photons PAR and a 16-8 h L-D cycle for several months during which the medium was regularly replaced. Acclimation to dynamic and constant irradiance was achieved by inoculating duplicate transparent polycarbonate erlenmeyers (2 L) with 30 mL culture, giving a total of 8 culture vessels. The dynamic irradiance set-up was as described in Van de Poll *et al.* (2007). Dynamic irradiance simulated vertical mixing over 4 h cycles, resulting in irradiance oscillations between 20-1350 μ mol photons \cdot m⁻² \cdot s⁻¹ (Fig. 1, see results), whereas constant irradiance was kept at 260 μ mol photons \cdot m⁻² \cdot s⁻¹ during the 16 h light period. The daily irradiance dose was similar for constant and dynamic irradiance. All cultures were maintained at 4.5°C by cryostat controlled water baths. After inoculation cell numbers were followed for 35 and 15 days, for iron-limited and replete cultures respectively. Iron-replete cultures were refreshed with new medium once. On the last day, samples for Fv/Fm, pigments, antioxidant activities and excessive irradiance sensitivity were obtained. After the experiments, the remaining iron-limited cultures were supplied with iron to test if this enhanced pigmentation, fluorescence and growth.

Growth

Two mL sub samples were obtained from the cultures for cell counts and immediately processed. Cell concentrations were determined on a Coulter MXL flow cytometer as in Van de Poll *et al.* (2006). The mean growth rate of each replicate was calculated from linear regression of 6–9 natural log transformed cell concentrations plotted against time.

Maximal quantum yield of PSII: Fv/Fm

To expand measuring capacity, two fluorometers were used to determine Fo and Fm after 5 min dark adaptation and the Fv/Fm was calculated as (Fm-Fo) / Fm. On the last day of the experiment, a dual modulated fluorometer with integrating sphere (Photosystem Instruments, Czech Republic, red excitation light; > 670 nm) was used to measure Fv/Fm of iron-replete and iron-limited cultures under dynamic irradiance. A pulse amplitude

modulation fluorometer (water PAM, Walz, Germany, blue excitation light; peak around 450 nm) was applied to measure Fv/Fm during excessive irradiance (simulated surface irradiance, SSI) in 5 mL sub samples, see below.

Pigment composition

Samples (75 mL) for pigment composition (one from each bottle, 2 replicates in total) were filtered on 25 mm GF/F (Whatman) by vacuum, frozen in liquid nitrogen and stored at -80°C. Filters were freeze-dried (48 h) followed by pigment extraction in 3 mL 90% cold acetone (v/v, 48 h, 4°C) after Van Leeuwe *et al.* (2006). Pigments were resolved by HPLC (Waters 2690 separation module, 996 photodiode array detector) with a C₁₈ 5 µm DeltaPak reversed-phase column (Waters) and identified by retention time and diode array spectroscopy. For quantification, standards of chlorophyll *a*, fucoxanthin, diadinoxanthin, and diatoxanthin were used (DHI, Denmark). Cellular pigment concentrations were calculated from cell counts (flow cytometer), sample volume and extraction volume. Chlorophyll *c*1, 2 and β carotene were detected but not quantified in the present study.

Antioxidant activity: SOD activity

100-200 mL of culture was filtered on polycarbonate filters (47 mm, 2 µm pore size) by vacuum and stored at -80°C for SOD activity analysis. The Riboflavin/NitroBlue Tetrazolium (RF/NBT) method Beauchamp & Fridovich 1971), modified by Janknegt *et al.* (2007), was used to measure SOD activity. Filters were suspended in 0.75 mL buffer (50 mM KH₂PO₄, pH 7.8, 0.1 mM EDTA, 0.1% Triton X-100, 2% PVP, and Complete protease inhibitor cocktail). Cells were disrupted by sonication (2 * 30 s pulses, 4°C), and extracts were centrifuged (20.000 g, 4°C, 25 min), and transferred to a clean vial. SOD activity was defined as the amount of sample required for 50% inhibition of NBT reduction (V_{50}). NBT reduction was measured in reaction mixtures (1.5 mL) with 0, 12.5, 25, and 50 µL extract, SOD buffer (50 mM KH₂PO₄, 0.1 mM EDTA, and 0.25% Triton X-100), 2 mM riboflavin (Sigma), and 57 µM NBT (Sigma). After 30 min dark incubation (4°C) the reaction mixtures were illuminated from above for 15 min (Philips TLD/18W, 199 µmol photons·m⁻²·s⁻¹). Afterwards, absorbance was measured at 560 nm on a Cary 3E UV/vis double beam spectrophotometer (Varian, Middelburg, The Netherlands). The V_{50} was calculated by regression using the linear part of a natural semi-log curve. The specific activity of SOD (U/mg protein) was calculated according to $1 / (V_{50} * [\text{protein}])$. Protein concentrations in the extracts were determined in duplicate according to Bradford (1976).

Antioxidant activity: APX and GR activity

Sampling, filtration and extract preparation for ascorbate peroxidase (APX) and glutathione reductase (GR) activities was as for the SOD activity (see above). APX activity was determined spectrophotometrically by the consumption of ascorbic acid, for 3 min in quartz cuvette containing 2.4 mL buffer (50 mM KH₂PO₄, pH 7; 0.1 mM EDTA), 150 µL 10 mM ascorbic acid (Merck, Darmstadt Germany), and 400 µL cell extract. Reaction mixtures were incubated (5 min) at 4°C before 20 mM H₂O₂ was added. The rate of ascorbate consumption was measured at 290 nm on a Cary 3E UV/vis double beam spectrophotometer at 4°C. Specific activity (U) of APX was calculated according to: $(-\text{slope} * \text{volume}_{\text{tot}} / \epsilon * \text{volume}_{\text{extr}}) / [\text{protein}]$ of which $\epsilon = 2.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

GR activity was determined by its ability to convert oxidized glutathione (GSSG) into its reduced form (GSH) using NADPH as a reducing agent. NADPH consumption was measured spectrophotometrically at 340 nm for 3 min in a 3 mL quartz cuvette with 1 mL buffer, 150 μ L, 10 mM GSSG (Merck, Darmstadt Germany), 300 μ L cell extract and 50 μ L, 2.5 mM NADPH (Merck, Darmstadt Germany). Before NADPH addition, the reaction mixture was incubated for 5 min at 4.5 °C. Specific activity of GR (in U/mg prot) was calculated according to: $(-\text{slope} * \text{volume}_{\text{tot}} / \epsilon * \text{volume}_{\text{extr.}}) / [\text{protein}]$ of which $\epsilon = 6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

Excessive irradiance sensitivity

350 mL portions of the cultures were transferred to 1N HCl washed quartz vessels and exposed to simulated surface irradiance (SSI: 448, 50, and 0,6 W m⁻² PAR, UVAR, and UVBR, respectively) as in Van de Poll *et al.* (2005). Excessive irradiance effects on pigment composition and Fv/Fm were examined by taking samples before, directly after 20 min of SSI, and after 20 min of SSI followed by 60 min in low (10 μ mol photons·m⁻²·s⁻¹) irradiance. In addition, viability loss during prolonged SSI exposure (4 h) was investigated in a separate experiment using 25 mL of culture for each replicate. Subsamples for the viability assay were obtained every hour during 4 h SSI exposure (see below). During SSI exposure temperature was maintained at 4.5°C.

Viability loss during excessive irradiance

One mL sub samples were incubated for 30 min in darkness with 10 μ L SYTOX (Molecular probes) solution that was 100 times diluted in MilliQ. SYTOX can enter and stain the DNA of cells with compromised membranes, a non-reversible condition that characterizes non-viable cells. SYTOX emits green fluorescence after excitation at 488 nm. The presence of cellular green fluorescence of SYTOX was assessed flow cytometrically. For each data point at least 30·10³ individual cells were analyzed.

Statistics

Differences between groups of replicates were tested for significance with a two factor (iron replete, iron limited, and constant, dynamic irradiance) ANOVA and a LSD post hoc test.

Results

Fv/Fm during dynamic irradiance

On the last day of the experiment, F_v/F_m was significantly higher for iron-replete than for iron-limited cells. Moreover, iron-replete cells showed a stronger response in F_v/F_m under dynamic irradiance than iron-limited cells (Fig. 1). For iron-limited cells, changes in F_v/F_m were minimal during the irradiance dynamics.

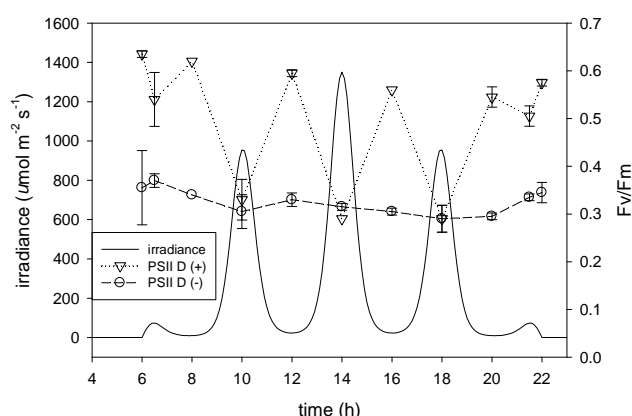


Figure 1: Irradiance and maximal quantum yield of PSII (F_v/F_m) of iron-limited (-) and replete (+) *Chaetoceros brevis* during the course of the dynamic (D) irradiance treatment. Mean and standard deviations are shown for two replicates.

Growth

Mean growth rates of iron-replete cultures (followed for 15 days) were significantly higher (~60%) than those of iron-limited cultures (followed for 35 days) ($p = 0.0001$, Fig. 2a). On the last day of the experiment all cultures were still in exponential growth. There was no significant difference in growth between static and dynamic irradiance under iron-limited and iron-replete conditions.

Pigment composition

Significant differences in pigment composition were found between iron-limited and iron-replete, and between dynamic and constant irradiance conditions. Total pigments (pg per cell) were 3 fold higher under iron-replete than iron-limited conditions for dynamic irradiance, whereas there was a twofold difference between iron-replete and iron-limited conditions under constant irradiance (Fig. 2b).

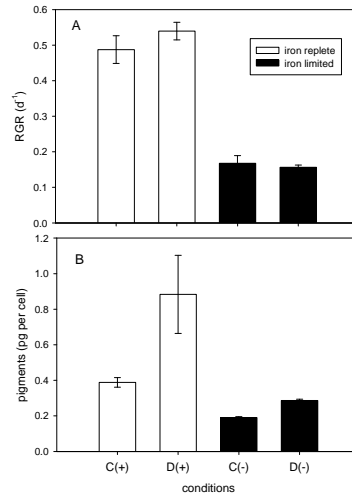


Figure 2: Relative growth rates (A) and cellular pigment concentrations (B) of iron-limited (-) and replete (+) *Chaetoceros brevis* grown under dynamic (D) and constant irradiance (C). Mean and standard deviations are shown for two replicates.

Cellular light harvesting (fucoxanthin and chlorophyll *a*) pigment contents were significantly higher for dynamic irradiance than for constant irradiance ($p=0.01$, Table 1). Cellular concentrations of both pigments were significantly lower during iron limitation ($p=0.005$). There was also a significant interaction between irradiance and iron availability for fucoxanthin ($p=0.036$) and chlorophyll *a* ($p=0.037$), showing different responses of the main light harvesting pigments under dynamic and constant irradiance during iron replete and limited conditions.

Table 1: Cellular concentrations of chlorophyll *a*, fucoxanthin, and the pool of diadinoxanthin and diatoxanthin of iron limited (-) and replete (+) *Chaetoceros brevis* grown under dynamic (D) and constant irradiance (C). The ratio between protective (diadinoxanthin, diatoxanthin) and light harvesting (chlorophyll *a*, fucoxanthin) is also shown (LP/LH). Mean and standard deviations are shown for two replicates.

Pigment	C (+)	D (+)	C (-)	D (-)
Chlorophyll <i>a</i>	0.130(0.007)	0.353(0.068)	0.048(0.002)	0.083(0.003)
Fucoxanthin	0.070(0.003)	0.198(0.037)	0.027(0.001)	0.053(0.004)
Diadino+diatoxanthin	0.093(0.015)	0.155(0.038)	0.058(0.008)	0.076(0.010)
Ratio (LP/LH)	0.470(0.079)	0.285(0.045)	0.783(0.139)	0.565(0.089)

The cellular pool of protective pigments (diadinoxanthin and diatoxanthin) was significantly different for irradiance and iron conditions. Iron-replete cells contained more

protective pigments than iron- limited cells, whereas cells grown under dynamic irradiance possessed a higher pool than those grown under constant irradiance. For the protective pigments there was no interaction between irradiance and iron conditions. The ratio of protective relative to light harvesting pigments was significantly higher for constant irradiance grown cells than for dynamic irradiance grown cells ($p=0.0001$), and was significantly higher for iron-limited cells compared to iron-replete cells. There was no interaction between irradiance and iron conditions for this ratio.

Antioxidant capacity

Significant differences in SOD and APX activities were not found between dynamic and constant irradiance grown cells under iron-replete conditions (Fig. 3). During iron limitation SOD and APX activity was increased 3 fold for cultures exposed to dynamic irradiance, but lower for those exposed to constant irradiance, compared to iron replete activity levels. APX activity followed a similar pattern as SOD activity, and the activity of both enzymes was significantly correlated ($r^2: 0.88$, $n = 8$, results not shown). Under iron limitation, APX activity was twofold enhanced under dynamic irradiance, but 10 fold reduced under constant irradiance. Significant differences in glutathione reductase were not found between any of the tested conditions (results not shown). The mean specific activity of glutathione reductase was $2.4 (\pm 0.4)$ U per mg protein for 8 replicates.

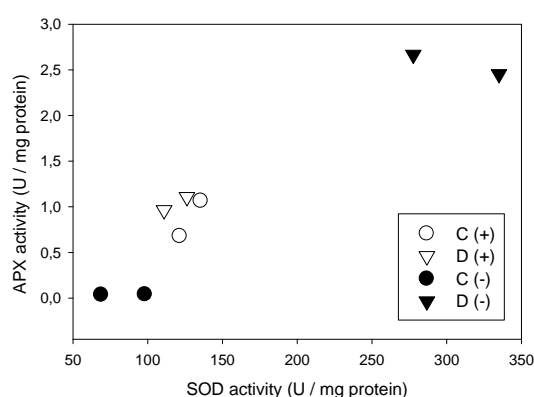


Figure 3: Activities of the antioxidant enzymes ascorbate peroxidase (APX) and superoxide dismutase (SOD) for *Chaetoceros brevis* grown under dynamic (D) and constant irradiance (C) under iron-limited (-) and replete (+) conditions. The activities were determined for two replicates.

Excessive irradiance sensitivity: F_v/F_m

Absolute F_v/F_m values were lower for the water PAM compared to those obtained with the PSI fluorometer, but the relative differences between the conditions were similar. As expected, there was a strong effect of iron on F_v/F_m : the highest F_v/F_m was found for iron-replete cells grown under dynamic irradiance. F_v/F_m was lower for iron-limited cells,

whereas significant differences between constant and dynamic irradiance cultivation were not observed (Figs. 4a, b). After 20 min SSI treatment Fv/Fm was significantly reduced to 23 and 58% of the pre-exposure values for iron-limited and replete conditions, respectively. Nearly complete recovery was found after 60 min in low irradiance for all cultivation conditions.

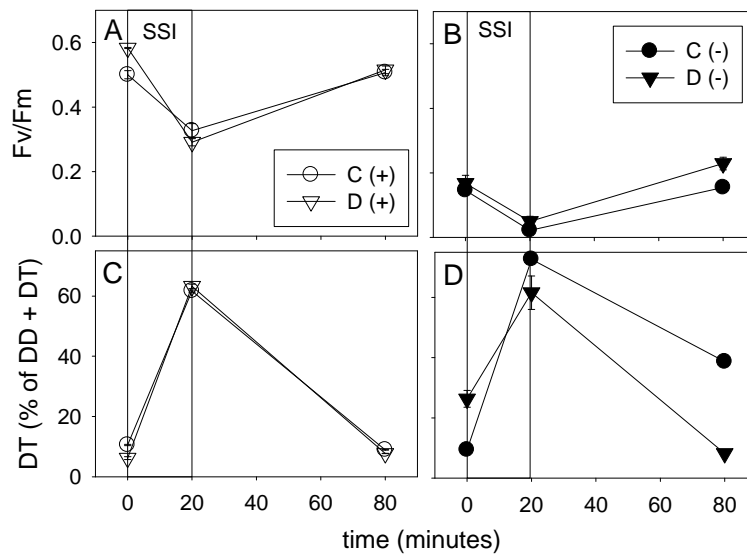


Figure 4: Dynamics of Fv/Fm (a, b) and the concentration of diatoxanthin relative to the diadino+diatoxanthin pool (c, d) during 20 min excessive irradiance exposure (SSI) and 60 min recovery in low irradiance for iron-replete (A, C) and limited (B, D) *Chaetoceros brevis* grown under dynamic (D) and constant irradiance (C). Mean and standard deviations are shown for two replicates.

Excessive irradiance sensitivity: Pigment composition

Apart from the de-epoxidation state of diadinoxanthin there were no significant changes in pigment composition after 20 min SSI and 60 min recovery in low irradiance (not shown). Before the SSI treatment 8-24% of the protective pigments was in the form of diatoxanthin (Figs. 4c, d). Directly after SSI this was 64-74%, whereas this was reduced to 8% after 60 min recovery in low irradiance, except for iron-limited cells that were grown in constant irradiance (39%).

Excessive irradiance sensitivity: Viability loss

The number of non-viable cells was low in iron-replete and iron-limited cultures before SSI exposure, with no difference between dynamic and static irradiance cultivation (mean $2.5 \pm 0.5\%$ of cells non-viable, results not shown). The proportion of non-viable cells did not change significantly during 4 h SSI exposure (4 h SSI: mean $2.5 \pm 0.9\%$ of cells non-viable).

Discussion

Chaetoceros brevis is a common open ocean species from the Southern Ocean with a low iron requirement due to its small size (5 μm) (Timmermans *et al.* 2001). Cultivation of this species under iron free conditions revealed some well described effects of iron limitation: a reduction in cellular pigments, a reduced ability to profit from irradiance, and highly reduced growth rates. During our experiment, we simulated vertical mixing by fluctuations between high (1350 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and low irradiance (20 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and compared growth and physiology of *C. brevis* with that under saturating, non fluctuating irradiance (260 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Obviously, irradiance patterns that algae experience in the water column are more complex than those during our treatments. Despite the large range in irradiance, no differences in growth rates were observed between the constant and dynamic treatments under iron-limited and replete conditions. Nevertheless, significant physiological differences were found in pigment composition, Fv/Fm, and antioxidant activity. This showed that the algae had photoacclimated efficiently to both conditions, thereby mitigating potential effects of irradiance limitation and photoinhibition. Furthermore, this agrees with recent findings for iron-limited diatom assemblages from the North Pacific that displayed clear physiological responses after iron addition, whereas changes in productivity were not observed (Hopkinson & Barbeau 2008).

The temporal irradiance fluctuations experienced during the dynamic irradiance treatment increased cellular pigment content more than threefold compared to constant irradiance, under iron-replete conditions. This is consistent with previous results for *Thalassiosira weissflogii* and *Emiliana huxleyi* grown under the same conditions (Van de Poll *et al.* 2007), showing that dynamic irradiance acclimated algae displayed more low irradiance acclimation characteristics than those acclimated to constant irradiance of equal daily dose. Furthermore, these results imply that vertical mixing increases cellular iron demand. Moreover it suggests that algae have a higher iron requirement under natural irradiance conditions (dynamics caused by diurnal cycle, position in the water column and weather) than under the constant irradiance conditions normally used in the lab. As previously demonstrated, reduced light availability increased the cellular demand of iron rich components (Raven 1990, Sunda & Huntsman 1997, Strzepec & Price 2001).

Differences in cellular pigment content and Fv/Fm between dynamic and constant irradiance were significantly smaller under iron limitation, suggesting a reduced acclimation potential to prevailing irradiance conditions. However, the absence of effects on growth of iron-limited cells during dynamic and constant irradiance indicated that other phenomena compensated for this reduction in light harvesting. For example, the disproportionate down-scaling of light harvesting capacity during dynamic irradiance and iron limitation could be compensated by reduced self-shading of pigments, thereby increasing the light harvesting efficiency of the remaining pigments. Therefore, the interaction between iron and irradiance treatments could have originated from reduced self shading as was also suggested by Geider *et al.* (1993). Furthermore, relatively high, saturating irradiance was applied in both treatments, whereas vertical mixing can reduce the irradiance that algae experience, and impose light limitation when algae are mixed below the euphotic zone. Possibly, differences between dynamic and constant irradiance would be more pronounced during iron limitation and light limitation.

Previously, it was suggested that iron limitation enhanced excessive irradiance effects such as photoinhibition (Geider *et al.* 1993, Strzepek & Harrison 2004, Van Oijen *et al.* 2005). However, our research suggests that iron-limited *C. brevis* was not more vulnerable to excessive irradiance exposure than under iron-replete conditions. Nevertheless, the activities of the antioxidant enzymes SOD and APX were markedly enhanced for iron-limited cells grown under dynamic irradiance. The increased activities of both enzymes is indicative of enhanced superoxide formation during dynamic irradiance. We propose that increased cellular antioxidant activity is triggered by the periodic over-reduction of the photosynthetic electron transport chain during fluctuations in PSII excitation. Evidence for elevated ROS during iron limitation was reported previously for diatoms (Peers & Price 2004). Elevated ROS may be due to impairment of the PSI and cytochrome b_6 -f complexes, which are particularly iron rich and their relative abundance can be reduced in response to iron limitation (Strzepek & Harrison 2004). An imbalance between PSII and cytochrome b_6 -f complexes could reduce the efficiency of the PSII electron cycle and therefore mediate increased over-reduction of PSII (Lavaud & Strzepek 2007). This scenario leads to enhanced superoxide radical formation, which requires increased scavenging by SOD and APX. In contrast to dynamic irradiance, a significant decrease in APX and SOD activities was found during iron limitation and constant irradiance. We hypothesize that the absence of irradiance fluctuations reduced PSII excitation pressure and therefore did not trigger elevated antioxidant activity. Because no growth reduction was found between dynamic and constant irradiance during iron limitation, it appeared that elevated antioxidant activity compensated the increased superoxide formation during the former condition. Elevated antioxidant activity can increase cellular iron demand because peroxidases like APX and superoxide dismutase (SOD) can contain iron cofactors. However, the iron cofactor in the latter enzyme can be substituted by manganese (Mn) under iron limitation in *T. weissflogii* (Peers & Price 2004). In our research SOD and APX activities were normalized to the protein content from the extracts. Normalization to chlorophyll *a* has been reported by Sunda *et al.* (2002), which would accentuate SOD and APX activity differences due to the large iron limitation mediated reduction in cellular chlorophyll *a*.

Iron-limited cells have a highly reduced light harvesting capacity, but this is accompanied by a much smaller reduction in the protective xanthophyll pigments pool. As a result, the ratio of protective relative to light harvesting pigments was higher during iron limitation of dynamic and constant irradiance grown cells. Previous experiments with low irradiance cultivated *C. brevis* showed pronounced viability loss during excessive irradiance. However, these cells had a 6 fold lower ratio of protective relative to light harvesting pigments than the cultures with the lowest ratio from the current experiment (iron-replete cells grown in dynamic irradiance) and a 16 fold difference with the iron-limited cells that were grown under constant irradiance. Consequently, *C. brevis* was relatively insensitive to the harmful effects of excessive irradiance under all of the current cultivation conditions, as shown by fast Fv/Fm recovery and the lack of viability loss after excessive irradiance.

Although the dynamic irradiance treatment covered almost the entire irradiance range that algae experience in the water column, Fv/Fm of iron-limited *C. brevis* showed surprisingly little response during the irradiance fluctuations. In contrast, Fv/Fm was strongly regulated under iron replete conditions during the dynamic irradiance regime, which coincided with xanthophyll (de-)epoxidation cycles (results not shown). The low

Fv/Fm under iron limitation at the start of the excessive irradiance exposure experiments coincided with low xanthophyll de-epoxidation. Therefore, low Fv/Fm values during iron limitation did not appear to be the result of xanthophyll cycle activity. The excessive irradiance exposure data showed no clear differences in xanthophyll cycle dynamics for iron-limited and replete cells after and during recovery from excessive irradiance. Furthermore, Fv/Fm recovery after 20 min simulated surface irradiance was not clearly different for iron replete and limited cells. Previous research on *Phaeocystis antarctica* reported reduced xanthophyll cycle activity under iron limitation during a dynamic irradiance treatment (Van leeuwe & Stefels 2007). Presumably, the proton gradient over the thylakoid membrane develops slower in iron-limited cells, thereby suppressing xanthophyll de-epoxidation. However, during our experiments we found no evidence for this and this issue should be verified in future research. The similar PSII response of iron limited and replete cells, and the lack of viability loss during excessive irradiance indicated that xanthophyll de-epoxidation could play a role in photo protection, regardless of iron availability.

In summary, PSII dynamics were reduced and SOD and APX activities were enhanced when iron-limited cells were grown under dynamic irradiance, in comparison to iron-replete cells. However, growth rates of iron-limited and replete *C. brevis* were not different under dynamic irradiance compared to saturating, non-fluctuating irradiance. This indicates that the increased antioxidant activity compensated for decreased non photochemical quenching efficiency around PSII. Furthermore, xanthophyll cycle activity was still induced in iron-limited cells under excessive irradiance. Thus, the observed physiological changes secured maintenance of growth during dynamic irradiance and photoprotection against excessive irradiance.

